cillin penetrates the growing cell more readily than the 'washed resting' cell. It would, however, seem equally possible that the loss of ability to oxidize guanylic acid following growth in the presence of penicillin is a secondary effect following some primary disorganization, an explanation put forward to explain the somewhat similar results obtained for the effect of penicillin on glutamic acid accumulation (Gale & Rodwell, 1949).

Such difficulties of interpretation do not arise in the cases of chloramphenicol, aureomycin and terramycin where the limiting concentration affecting protein synthesis is the same as that affecting growth.

SUMMARY

1. Chloramphenicol, aureomycin and terramycin inhibit protein synthesis by washed suspensions of

Staphylococcus aureus, at bactericidal concentrations. Such concentrations simultaneously stimulate nucleic acid synthesis.

2. Bacitracin inhibits protein and nucleic acid synthesis to the same extent. Complete inhibition requires a concentration of bacitracin 100 times that which prevents growth.

3. Penicillin in high concentrations prevents the acceleration of protein synthesis produced by the addition of purines and pyrimidines to the incubation mixture containing amino-acids and glucose.

The authors are indebted to Miss P. J. Samuels for assistance with chromatographic procedures and to Dr T. F. Paine for gifts of aureomycin, streptomycin and bacitracin.

REFERENCES

- Caldwell, P. C. & Hinshelwood, C. (1950). *J. chem. Soc.* p. 3156.
 Chantrenne, H. (1952). Symp. Soc. gen. Microbiol: *The Nature of Virus Multiplication*. Cambridge University Press.
 Gale, E. F. (1947a). *J. gen. Microbiol.* **1**, 53.
 Gale, E. F. (1947b). *J. gen. Microbiol.* **1**, 327.
 Gale, E. F. (1951a). *Biochem. J.* **48**, 286.
 Gale, E. F. (1951b). *Biochem. J.* **48**, 290.
 Gale, E. F. & Folk, J. P. (1953). *Biochem. J.* **53**, 483.
 Gale, E. F. & Paine, T. F. (1951). *Biochem. J.* **48**, 298.
 Gale, E. F. & Rodwell, A. W. (1949). *J. gen. Microbiol.* **3**, 128.
 Gale, E. F. & Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 314.
 Gale, E. F. & Van Halteren, M. B. (1951). *Biochem. J.* **50**, 34.
 Gros, F., Beljanski, M. & Macheboeuf, M. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1696.
 Gros, F. & Macheboeuf, M. (1948a). *Ann. Inst. Pasteur*, **74**, 308.
 Gros, F. & Macheboeuf, M. (1948b). *Bull. Acad. Méd., Paris*, **5**, 80.
 Hotchkiss, R. D. (1949). *Fed. Proc.* **8**, 208.
 Hotchkiss, R. D. (1950). *J. exp. Med.* **91**, 351.
 Hotchkiss, R. D. (1952). 2nd International Congress of Biochemistry Symposium: *Mode d'action des antibiotiques*, p. 40.
 Krampitz, L. O. & Werkman, C. H. (1947). *Arch. Biochem.* **12**, 57.
 Loomis, W. F. (1950). *Science*, **111**, 474.
 Mitchell, P. D. & Moyle, J. (1951). *J. gen. Microbiol.* **5**, 421.
 Paine, T. F. jun. (1951). *J. Bact.* **61**, 259.
 Park, J. T. (1952). *J. biol. Chem.* **194**, 877, 885, 897.
 Park, J. T. & Johnson, M. (1949). *J. biol. Chem.* **179**, 585.
 Pollock, M. R. (1950). *Brit. J. exp. Path.* **31**, 739.

The Oxidation of Certain Dicarboxylic Acids by Peroxidase Systems in Presence of Manganese

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(Received 9 August 1952)

It has frequently been suggested that the physiological effects of manganese may be due to its capacity for valency change. Kenten & Mann (1950) have shown that Mn^{2+} is oxidized by peroxidase systems. Such oxidation takes place in plant extracts (Kenten & Mann, 1951) and preliminary results suggest that it also occurs *in vivo* in higher plants. Kenten & Mann (1949) suggested

that the manganese oxidation product reacts with plant metabolites whereby the Mn^{2+} is involved in an oxidation-reduction cycle which could explain its effect on plant respiration. As yet, however, there is little evidence as to the nature of the metabolites oxidized.

A number of instances have been recorded where an activating effect of Mn^{2+} on oxidative reactions is

associated with the presence of peroxidase or compounds possessing peroxidatic activity. Thus the rate of oxidation of dihydroxymaleic acid by cytochrome *c* or peroxidase is increased by Mn^{2+} (Theorell & Swedin, 1939; Swedin & Theorell, 1940). It is well known that the spontaneous and enzyme-catalysed decarboxylations of oxaloacetate and oxalosuccinate are accelerated by a variety of polyvalent cations; Mn^{2+} is usually the most effective cation for the enzyme-catalysed reactions (Krebs, 1942; Speck, 1949; Kornberg, Ochoa & Mehler, 1948; Gollub & Vennesland, 1947; Vennesland, Ceithaml & Gollub, 1947). In some cases an oxidative decarboxylation of oxaloacetate and oxalosuccinate has been found when Mn^{2+} has been used to activate the enzyme preparations from plant tissues (Vennesland & Felsher, 1946; Ceithaml & Vennesland, 1949). Vennesland & Evans (1944) found that oxaloacetic acid was oxidatively decarboxylated to malonic acid by a preparation from heart muscle in presence of Mn^{2+} . Vennesland, Evans & Francis (1946) found that the active factor in such preparations was metmyoglobin and pointed out the similarity between the reaction and the oxidation of dihydroxymaleic acid catalysed by cytochrome *c* and Mn^{2+} . Since it is now known that peroxidase systems oxidize Mn^{2+} it appeared possible that the effect of Mn^{2+} in these systems was due to its oxidation through peroxidase or the peroxidatic action of metmyoglobin and cytochrome *c*. The present work was undertaken to investigate this possibility.

MATERIALS AND METHODS

Peroxidase and catalase preparations. These were as described by Kenten & Mann (1952).

Plant saps. The plant material was washed free from soil, ground in a mortar with sand and squeezed through madapollam.

Oxaloacetate and dihydroxytartrate solutions. These were prepared immediately before use from oxaloacetic and dihydroxytartaric acids and adjusted to the required pH with *N*-NaOH.

Ketomalonalate solutions. These were made from disodium ketomalonalate and adjusted to the required pH with *m*-acetic acid.

Dihydroxymaleate solutions. These were prepared immediately before use by dissolving dihydroxymaleic acid in 0.05 *M*-acetate buffer, pH 5, and adjusting to pH 5 with *N*-NaOH.

Dihydroxymaleic acid. This was prepared by the action of H_2O_2 on tartaric acid according to the method of Fenton (1894). The recrystallized product decomposed at 152° (uncorr.). Fenton (1894) gives the decomposition temperature as about 155°.

Manometric measurements. These were made in the Warburg apparatus at 25°. The volume of the reaction mixtures was 3 ml. O_2 uptakes were measured directly with KOH in the centre cups. CO_2 outputs were measured by Warburg's direct method (Dixon, 1943).

EXPERIMENTAL AND RESULTS

The oxidation of certain dicarboxylic acids by peroxidase systems in presence of Mn^{2+}

Kenten & Mann (1952) showed that manganese oxidation could be brought about by coupling enzyme systems producing H_2O_2 with peroxidase systems. An increased O_2 uptake was obtained when oxalate was added to such systems in presence but not in absence of Mn^{2+} . This was attributed to a manganese oxidation-reduction cycle, but the results obtained suggested that reactions were proceeding in addition to the coupled oxidation of the manganese by the enzyme system followed by reduction of the oxidized manganese by the oxalate. Launer (1933) showed that when Mn^{3+} was added to oxalate solutions the CO_2 output in air was considerably greater than that required for complete reduction of the Mn^{3+} to Mn^{2+} by the oxalate. Evidence was obtained that this was due to re-oxidation of the Mn^{2+} accompanied by H_2O_2 formation. In the present work experiments were made to investigate whether such H_2O_2 formation affected the interpretation of the results of Kenten & Mann (1952). It was found that, while reaction mixtures containing oxalate and Mn^{2+} alone showed no O_2 uptake, the addition of peroxidase together with *p*-cresol to such reaction mixtures caused an O_2 uptake after an initial lag period. The rate of O_2 uptake depended on the concentration of Mn^{2+} . A typical set of curves is shown in Fig. 1. Reaction mixtures consisted of 100 μ g. peroxidase of purpurogallin number (P.Z.) 250 (Willstätter & Stoll, 1918), 0.3 ml. 0.001 *M*-*p*-cresol and 0.5 ml. 0.1 *M*-sodium oxalate, pH 5, with 1 ml. 0.1 *M*-acetate buffer, pH 5. Manganese sulphate (0.2 ml. 0.1 *M* or 0.01 *M*) was added from the side arm after equilibration. Control experiments were made in which peroxidase, *p*-cresol or Mn^{2+} were omitted.

The fact that the O_2 uptake is dependent on the presence of peroxidase and *p*-cresol suggests that H_2O_2 formation and manganese oxidation are involved in the reaction mechanism. With the higher Mn^{2+} concentration a faint pink colour was present in the reaction mixture at the end of the lag period. This colour became more intense during the period of rapid O_2 uptake and then decreased in intensity. The pink colour is probably due to the presence of a manganioxalate complex such as those described by Cartledge & Ericks (1936). The lag period could be reduced by adding small amounts of H_2O_2 (0.1 ml. 0.005 *M*) from the side arm, a fact which supports the suggestion that H_2O_2 is involved in the reaction. As would be expected, the added H_2O_2 caused oxidation of the Mn^{2+} and the reaction mixture rapidly became pink owing to the formation of manganio-

oxalate. It appears, therefore, that the lag period may be due to the slow build-up of manganioxalate. Once sufficient of this is present the O_2 uptake and manganioxalate formation proceed rapidly. Manganioxalate could be rapidly formed in the reaction mixtures by adding small amounts of hydrated MnO_2 (e.g. 50 $\mu g.$ MnO_2) which were reduced by the Mn^{2+} present to the Mn^{3+} state. Such additions of MnO_2 also reduced the lag period.

The O_2 uptake was accompanied with a CO_2 output and at the end of the experimental period R.Q.'s of about 3.5 were obtained. It is therefore clear that the greater part of the observed O_2 uptake represents oxidation of the oxalate.

Fig. 1. Catalysis of the oxidation of oxalate by peroxidase, Mn^{2+} and *p*-cresol. Complete system with 1100 $\mu g.$ Mn^{2+} : $\square-\square$, no H_2O_2 ; $\triangle-\triangle$, primed with 0.1 ml. 0.005M- H_2O_2 . Complete system with 110 $\mu g.$ Mn^{2+} : $\circ-\circ$, no H_2O_2 ; $+-+$, primed with 0.1 ml. 0.005M- H_2O_2 . Controls with 1100 $\mu g.$ Mn^{2+} : $\blacktriangle-\blacktriangle$, peroxidase omitted; $\times-\times$, *p*-cresol omitted; $\bullet-\bullet$, Mn^{2+} omitted.

It appeared unlikely that the oxalate reaction could be of physiological importance owing to the relatively high concentrations of Mn^{2+} required. Since it was improbable that the reaction was specific for oxalate a variety of carboxylic acids were tested under similar conditions. Negative results were obtained with citric, aconitic, tartaric, malic, acetoacetic and α -ketoglutaric acids in manometric experiments though, in some cases, the reaction mixtures gave positive colorimetric tests with benzidine suggesting the presence of H_2O_2 or of oxidized manganese. In manometric experiments positive results were obtained with oxaloacetic, ketomalonic and dihydroxytartaric acids.

Oxaloacetate

With oxaloacetate it was found that the reaction proceeded rapidly at much lower concentrations of Mn^{2+} than with oxalate. A typical set of results is illustrated in Fig. 2. Reaction mixtures were made up as for the experiments of Fig. 1 except that the oxalate was replaced by oxaloacetate (3 mg. oxaloacetic acid) and 0.05, 0.1 and 0.2 ml. 0.01M- $MnSO_4$ were added from the side arms after equilibration. The lag period was about 15 min., instead of 80 min. in the case of oxalate, after which a

Fig. 2. Catalysis of the oxidation of oxaloacetate by peroxidase, Mn^{2+} and *p*-cresol. Complete system: $\square-\square$, 110 $\mu g.$ Mn^{2+} ; $\circ-\circ$, 55 $\mu g.$ Mn^{2+} ; $\triangle-\triangle$, 27.5 $\mu g.$ Mn^{2+} ; $+-+$, 110 $\mu g.$ Mn^{2+} primed with 0.1 ml. 0.005M- H_2O_2 . Controls with 110 $\mu g.$ Mn^{2+} : $\bullet-\bullet$, peroxidase omitted; $\times-\times$, *p*-cresol omitted; $\blacktriangle-\blacktriangle$, Mn^{2+} omitted.

comparatively rapid O_2 uptake took place, the rate and total uptake increasing with the amount of Mn^{2+} added. The lag period was shortened by addition of 0.1 ml. 0.005M- H_2O_2 . The uptake depended on the presence of both peroxidase and *p*-cresol and was far greater than that required for the oxidation of the Mn^{2+} added. The effect of the Mn^{2+} was therefore to catalyse the oxidation of the oxaloacetate. No visual evidence was obtained during the experiment of the accumulation of manganic complexes. But on subsequent addition of benzidine an immediately positive test was obtained only with the complete reaction mixture. This suggests that the positive test was due to oxidized manganese rather than H_2O_2 . The O_2

uptake was accompanied by an increased CO_2 output (Fig. 3). Oxaloacetate alone undergoes spontaneous decarboxylation under the experimental conditions. The rate of CO_2 output was increased by Mn^{2+} alone (Krebs, 1942; Speck, 1949) but much more markedly by Mn^{2+} where both peroxidase and *p*-cresol were also present. Peroxidase and *p*-cresol alone, in the absence of Mn^{2+} , did not affect the spontaneous decarboxylation of oxaloacetate.

Fig. 3. CO_2 output and O_2 uptake during catalysis of the oxidation of oxaloacetate by peroxidase, Mn^{2+} and *p*-cresol. CO_2 outputs: $\times-\times$, complete system; $\bullet-\bullet$, peroxidase omitted; $+-+$, Mn^{2+} omitted. O_2 uptakes: $\blacksquare-\blacksquare$, complete system; $\blacktriangle-\blacktriangle$, peroxidase omitted.

Ketomalonate

The reaction with ketomalonate was studied in some detail since it proceeds very readily and is not complicated by loss of substrate by spontaneous decarboxylation.

Effect of variation in Mn^{2+} concentration on O_2 uptake. This is shown by the typical set of curves of Fig. 4. Reaction mixtures were as for Fig. 2, except that oxaloacetate was replaced by ketomalonate (3 mg. disodium ketomalonate) and amounts of MnSO_4 varying from 0.1 ml. 0.001M to 0.1 ml. 0.01M were added from the side arms. With the highest amount of Mn^{2+} added (110 μg .) there was a lag period of about 40 min. The lag period decreased with decrease in Mn^{2+} addition. While the length of the lag period varied in different experiments its

dependence on the concentration of Mn^{2+} was always observed. The lag period with 110 μg . Mn^{2+} was almost completely eliminated by adding 0.1 ml. 0.005M- H_2O_2 to the reaction mixture. In contrast to the results with oxaloacetate it was found that the oxidation of ketomalonate proceeded, though at a much reduced rate, when *p*-cresol was omitted from the reaction mixtures. At the end of the experiment positive benzidine tests were obtained in the complete reaction mixture and also where *p*-cresol was omitted. The O_2 uptake was accompanied by a CO_2 output as shown by the results of Fig. 5. Under the conditions used ketomalonate does not undergo

Fig. 4. Catalysis of the oxidation of ketomalonate by peroxidase, Mn^{2+} and *p*-cresol. Complete system: $\bigcirc-\bigcirc$, 110 μg . Mn^{2+} ; $+-+$, 27.5 μg . Mn^{2+} ; $\times-\times$, 5.5 μg . Mn^{2+} ; $\triangle-\triangle$, 110 μg . Mn^{2+} primed with 0.1 ml. of 0.005M- H_2O_2 . Controls: $\square-\square$, 110 μg . Mn^{2+} , *p*-cresol omitted; $\bullet-\bullet$, Mn^{2+} omitted, primed with 0.1 ml. 0.005M- H_2O_2 .

spontaneous decarboxylation and the correlation of the CO_2 output with the O_2 uptake was therefore clearer than with oxaloacetate.

Effect of variation of peroxidase concentration and addition of catalase. Reaction mixtures to test the effect of variation in peroxidase concentration were as for Fig. 4 except that 0.1 ml. of 0.01M- MnSO_4 (55 μg . Mn^{2+}) was present throughout and varying amounts of peroxidase (0–100 μg . of P.Z. 250) were added. A measurable O_2 uptake was obtained in presence of 2 μg . peroxidase and the maximum velocity was reached at about 20 μg . Further increase in peroxidase up to 100 μg . produced little change in this velocity. At the lower concentrations of peroxidase little or no O_2 uptake was observed

when the *p*-cresol was omitted. The O_2 uptake was decreased in rate but not completely eliminated by heat treatment (15 min. in boiling-water bath) of the peroxidase. The effect of this heat treatment was greater with the lower amounts of peroxidase. Catalase prolonged the lag period but did not completely inhibit the oxidation under the conditions used. Thus in one experiment where the reaction was catalysed by 10 μ g. peroxidase (P.Z. 250) the lag period was increased by 10 and 40 min. by 0.1 and 1.0 mg. catalase ($k'_1 = 2.21 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$; Chance & Herbert, 1950) respectively.

Fig. 5. CO_2 output and O_2 uptake during catalysis of the oxidation of ketomalonate by peroxidase, Mn^{2+} and *p*-cresol. CO_2 outputs: x—x, complete system; Δ — Δ , peroxidase omitted. O_2 uptakes: ■—■, complete system; Δ — Δ , peroxidase omitted.

Effect of varying ketomalonate concentrations. This was studied in reaction mixtures as for Fig. 4 except that 20 μ g. peroxidase of P.Z. 250 were used and the additions of ketomalonate varied between 0.3 and 3.0 mg. The reaction proceeded readily at all the concentrations of ketomalonate used and the total uptakes were roughly proportional to the amounts present. The lag period decreased with decreasing concentration of ketomalonate from about 10 min. with 1 mg./ml. to 2–3 min. with 0.1 mg./ml. For this reason it is difficult to correlate the initial rate of O_2 uptake with ketomalonate concentration.

Effect of addition of metallic ions other than Mn^{2+} . The effect on the oxidative decarboxylation of oxaloacetate and ketomalonate of substituting Co^{2+} , Zn^{2+} , Ni^{2+} , Al^{3+} , Fe^{2+} and Fe^{3+} for Mn^{2+} was investigated under the conditions of Fig. 4. Additions of these ions were made at two levels to give final concentrations of 0.066 and 0.0066M. No appreciable O_2 uptake was observed except with Fe^{2+} at the higher concentration. This uptake was unaffected by the presence of peroxidase and *p*-cresol, and was approximately equal to that required for the oxidation of the Fe^{2+} to Fe^{3+} . It was therefore concluded that the O_2 uptake was due solely to this oxidation and that the oxidation of diketomalonate was not catalysed.

Dihydroxytartrate

Each reaction mixture contained 3 mg. sodium dihydroxytartrate in 0.033M-acetate buffer at pH 5 and, where present, 0.4 ml. 0.001M- $MnSO_4$, 0.3 ml. 0.001M-*p*-cresol and 10 or 100 μ g. peroxidase (P.Z. 250). At this pH solutions of dihydroxytartrate undergo rapid spontaneous decarboxylation. The decarboxylation is accompanied by a comparatively slow O_2 uptake. The rate of CO_2 output was increased by Mn^{2+} though little effect was produced on the rate of O_2 uptake. Peroxidase alone had little or no effect on the rate of O_2 uptake in absence of Mn^{2+} but in presence of Mn^{2+} caused a rapid uptake. The rate of this uptake was only clearly increased by *p*-cresol at the lower level of peroxidase. Here, during the earlier part of the reaction period, *p*-cresol produced increases of 50–100%. Owing to the rapid spontaneous decarboxylation of the dihydroxytartrate solutions it is not clear how far the oxidation observed is that of dihydroxytartrate or its decarboxylation product.

Dihydroxymaleate

Under the conditions used with oxaloacetate, ketomalonate and dihydroxytartrate the oxidation of dihydroxymaleate is rapidly catalysed by peroxidase in presence of Mn^{2+} and the rate is further increased when *p*-cresol also is present. But the results are difficult to interpret since solutions of dihydroxymaleate alone show a small O_2 uptake, the rate of which is increased by peroxidase and still further by peroxidase together with *p*-cresol. Also, Mn^{2+} alone causes a large increase in the rate of O_2 uptake. A clearer picture is obtained if the reactions are carried out in presence of catalase which markedly reduces the increased rate of O_2 uptake produced by Mn^{2+} alone. The results of an experiment in presence of catalase are shown in Fig. 6. Reaction mixtures consisted of 2 mg. dihydroxymaleate in 0.66M-acetate buffer at pH 5, 0.1 mg. catalase, 0.1 ml. 0.001M- $MnSO_4$ (5.5 μ g. Mn^{2+}) and 0.3 ml. 0.001M-*p*-cresol. The Mn^{2+} and per-

oxidase were added from the side arms after equilibration. Simultaneous addition of Mn^{2+} and peroxidase produced an increase in the initial rate of O_2 uptake much greater than the sum of the increases in rate produced by these factors separately. *p*-Cresol, where present in addition to Mn^{2+} and peroxidase, produced a further increase in rate which was greater than the increase produced by *p*-cresol with peroxidase alone. At the end of the experiment all the reaction mixtures gave negative

coloration was discharged by hydrazine with gas evolution and was presumable due to manganipyrrophosphate (Kenten & Mann, 1949).

Oxidation of oxaloacetate and ketomalonate by pea-seedling sap with added Mn^{2+} . Kenten & Mann (1952) have shown that Mn^{2+} is oxidized by saps from many plants by the peroxidase systems present. These

Fig. 6. Catalysis of the oxidation of dihydroxymaleate by peroxidase Mn^{2+} and *p*-cresol. ○—○, complete system; □—□, *p*-cresol omitted; △—△, peroxidase and *p*-cresol omitted; +—+, Mn^{2+} omitted; ●—●, Mn^{2+} and *p*-cresol omitted; ▲—▲, peroxidase, Mn^{2+} and *p*-cresol omitted.

tests with benzidine showing that no manganese oxidation product had accumulated. But in pyrophosphate media using relatively high concentrations of $MnSO_4$ colorimetric evidence was obtained of the accumulation of manganipyrrophosphate in reaction mixtures containing, in addition, dihydroxymaleate, peroxidase and *p*-cresol. The reaction mixtures were made up in beakers and consisted of 0.5 ml. 0.2M-pyrophosphate buffer at pH 7 containing 0.2 mg. dihydroxymaleic acid and, where present, 0.2 ml. 0.1M- $MnSO_4$, 100 μg. peroxidase of P.Z. 250 and 0.3 ml. 0.001M-*p*-cresol. Water was added to give final volumes of 3 ml. A pink coloration appeared in the complete reaction mixture after about 15 min. at room temperature. This pink

Fig. 7. Oxidation of oxaloacetate by pea-seedling sap in presence of Mn^{2+} . ○—○, sap alone or sap + 110 μg. Mn^{2+} ; △—△, sap and oxaloacetate; ■—■, sap and oxaloacetate + 27.5 μg. Mn^{2+} ; ●—●, sap and oxaloacetate + 110 μg. Mn^{2+} .

saps with added Mn^{2+} would therefore be expected to oxidize oxaloacetate and ketomalonate. Such oxidation could readily be demonstrated with pea-seedling sap though negative results were obtained with tobacco-leaf sap. Results with pea-seedling sap are illustrated by the curves of Figs. 7 and 8. In these experiments reaction mixtures consisted of 0.5 ml. sap from 7- to 8-day-old pea seedlings and 1 ml. 0.1M-acetate buffer at pH 5 with additions of Mn^{2+} varying from 0.1 ml. of 0.001M to 0.2 ml. of 0.01M- $MnSO_4$. Oxaloacetic acid (3 mg.) or disodium ketomalonate (3 mg.) were added from the side arms after equilibration. A slight output was observed when the oxaloacetate was added and the subsequent apparent O_2 uptake was very slow over

the following 15–20 min. After this time oxaloacetate had little effect on the O_2 uptake of the sap in absence of added Mn^{2+} , but in presence of added Mn^{2+} a markedly increased O_2 uptake succeeded the lag period. The rate of this uptake depended on the amount of Mn^{2+} added. In presence of ketomalonate no lag period was observed and the rate of O_2 uptake was clearly increased by additions of $MnSO_4$ as low as 0.1 ml. of 0.001M ($5.5 \mu g.$ Mn^{2+}). The O_2 uptake in presence and absence of ketomalonate and Mn^{2+} was completely eliminated by heating the sap (15 min. in a boiling-water bath).

Fig. 8. Oxidation of ketomalonate by pea-seedling sap in presence of Mn^{2+} . +—+, sap alone; $\triangle$ — $\triangle$, sap + ketomalonate; $\circ$ — $\circ$, sap + $22 \mu g.$ Mn^{2+} ; $\square$ — $\square$, sap + ketomalonate + $5.5 \mu g.$ Mn^{2+} ; $\blacksquare$ — $\blacksquare$, sap + ketomalonate + $11 \mu g.$ Mn^{2+} ; $\bullet$ — $\bullet$, sap + ketomalonate + $22 \mu g.$ Mn^{2+} . $\blacktriangle$ — $\blacktriangle$, boiled sap + ketomalonate; $\times$ — $\times$, boiled sap + ketomalonate + $22 \mu g.$ Mn^{2+} .

DISCUSSION

The results of the present work show that Mn^{2+} in presence of peroxidase or peroxidase systems can catalyse the oxidation of certain dicarboxylic acids by O_2 . At the concentrations of peroxidase used, a peroxidase substrate such as *p*-cresol is necessary for the oxidation of oxalate and oxaloacetate. It has already been shown that peroxidase in presence of *p*-cresol catalyses the oxidation of Mn^{2+} by H_2O_2 (Kenten & Mann, 1950). It is therefore reasonable to suggest that the observed oxidation of these two

acids is dependent on manganese oxidation. With oxalate, colorimetric evidence suggests the accumulation of manganiooxalate during the early stages of rapid oxidation. With oxaloacetate, accumulation of manganic complexes is not apparent, but the reaction mixtures containing Mn^{2+} give strongly positive tests with benzidine suggesting the presence of H_2O_2 or of oxidized manganese. The reactions depend on the presence of peroxidase and the lag period can be shortened by adding H_2O_2 or increased by catalase. Hydrogen peroxide is therefore presumably formed during the reactions. It is suggested that the manganio-oxalate autoxidizes very slowly under the experimental conditions giving traces of H_2O_2 . This results in the formation of manganic manganese either during the autoxidation or in the peroxidase-catalysed oxidation of the manganio-oxalate by the H_2O_2 so produced. Launer (1933) has shown that H_2O_2 is formed during the oxidation of oxalate by manganic manganese in the presence of O_2 . Once manganic manganese is formed, therefore, further H_2O_2 will be produced presumably by the mechanism postulated by Launer (1933). In agreement with this is the finding that the lag period can be shortened by addition of MnO_2 .

The dependence of the oxidation of ketomalonate, dihydroxytartrate and dihydroxymaleate on the presence of peroxidase substrates such as *p*-cresol is less marked. Thus the oxidation of ketomalonate proceeds, though at a comparatively slow rate, in absence of *p*-cresol provided the peroxidase concentration is sufficiently high. The oxidation of dihydroxytartrate and dihydroxymaleate proceeds at all the concentrations of peroxidase used in the absence of *p*-cresol, though the addition of *p*-cresol increases the rate of oxidation at low peroxidase levels. It is possible that the oxidation of manganic complexes of these acids is directly catalysed by peroxidase. In this connexion it is of interest that peroxidase directly catalyses the oxidation of ferrocyanide, though at a much lower rate than in the presence of *p*-cresol (Kenten & Mann, 1951). The accumulation of manganic complexes of ketomalonate and dihydroxytartrate was not apparent during the oxidations, but positive benzidine tests were obtained during the oxidation of ketomalonate. It should be pointed out that these acids reduce MnO_2 much more rapidly than oxalic acid does under the experimental conditions. When the oxidation of dihydroxymaleate by the system peroxidase, Mn^{2+} and *p*-cresol was carried out in pyrophosphate buffer at pH 7, manganipyrophosphate accumulated. The oxidation of dihydroxymaleate differs from that of other compounds used in that it is catalysed by addition of peroxidase alone or of Mn^{2+} alone (Szent-Gyorgyi, 1939; Swedin & Thorell, 1940).

On the basis of these results it is suggested that the previously reported oxidations of dihydroxymaleate and oxaloacetate dependent on the presence of both Mn^{2+} and peroxidase or compounds with peroxidatic activity (Theorell & Swedin, 1939; Swedin & Theorell, 1940; Vennesland *et al.* 1946) involves the oxidation of Mn^{2+} . Likewise, it is suggested that the reported oxidation of oxaloacetate and oxalosuccinate by plant-tissue preparation in presence of Mn^{2+} (Vennesland & Felsher, 1946; Ceithaml & Vennesland, 1949) is due to the presence of peroxidase and depends on the oxidation of Mn^{2+} .

It is well known since the early work of Thurlow (1925) and Harrison & Thurlow (1926) that peroxidase can catalyse the oxidation of its substrates with H_2O_2 produced by flavoprotein enzyme systems. The systems studied in the present work, however, are of particular interest since they are independent of external sources of H_2O_2 . How far such systems are of significance *in vivo* is not yet known. In some cases the reactions investigated proceed rapidly at physiological concentrations of Mn^{2+} , but the substrates oxidized in these cases are not known as intermediates in metabolism. However, only a few compounds have as yet been tested in the system. The requirements of the system for Mn^{2+} in the oxidation of oxaloacetate are rather high in relation to normal physiological concentrations of Mn^{2+} . It therefore seems doubtful whether this oxidation is of significance *in vivo* except possibly under

conditions of manganese toxicity particularly as the oxidation product, presumably malonic acid, is known to inhibit the tricarboxylic acid cycle.

SUMMARY

1. The oxidation of oxalate, oxaloacetate, keto-malonate and dihydroxytartrate by oxygen is catalysed by peroxidase systems in presence of Mn^{2+} .

2. It is suggested that these oxidations and that of dihydroxymaleate in presence of peroxidase systems and Mn^{2+} involves the oxidation of Mn^{2+} .

Note. In a paper, which appeared after the present work was submitted for publication, Chance (1952) put forward an explanation of the effect of Mn^{2+} on the oxidation of dihydroxymaleic acid based on the activating effect of Mn^{2+} ions on a peroxidase-hydrogen peroxide complex and not dependent on manganese oxidation. If this explanation is correct the manganese oxidation observed on the present work in the system peroxidase-dihydroxymaleic acid- Mn^{2+} is a side reaction and dependent on the high concentration of Mn^{2+} used. In view of the results obtained with other substrates, however, it seems reasonable to suggest that manganese oxidation is not a side reaction and that the absence of accumulated manganese oxidation products at low Mn^{2+} concentration is due to their rapid reduction by the dihydroxymaleic acid.

REFERENCES

- Cartledge, G. H. & Ericks, W. P. (1936). *J. Amer. chem. Soc.* **58**, 2061.
- Ceithaml, J. & Vennesland, B. (1949). *J. biol. Chem.* **178**, 133.
- Chance, B. (1952). *J. biol. Chem.* **197**, 577.
- Chance, B. & Herbert, D. (1950). *Biochem. J.* **46**, 402.
- Dixon, M. (1943). *Manometric Methods*, 2nd ed. p. 64. Cambridge University Press.
- Fenton, H. J. H. (1894). *J. chem. Soc. Trans.* **65**, 899.
- Gollub, M. C. & Vennesland, B. (1947). *J. biol. Chem.* **169**, 233.
- Harrison, D. C. & Thurlow, S. (1926). *Biochem. J.* **20**, 217.
- Kenten, R. H. & Mann, P. J. G. (1949). *Biochem. J.* **45**, 255.
- Kenten, R. H. & Mann, P. J. G. (1950). *Biochem. J.* **46**, 67.
- Kenten, R. H. & Mann, P. J. G. (1951). *Biochem. J.* **50**, 360.
- Kenten, R. H. & Mann, P. J. G. (1952). *Biochem. J.* **52**, 125.
- Kornberg, A., Ochoa, S. & Mehler, A. H. (1948). *J. biol. Chem.* **174**, 159.
- Krebs, H. A. (1942). *Biochem. J.* **36**, 303.
- Launer, H. F. (1933). *J. Amer. chem. Soc.* **55**, 865.
- Speck, J. F. (1949). *J. biol. Chem.* **178**, 315.
- Swedin, B. & Thorell, H. (1940). *Nature, Lond.*, **145**, 71.
- Szent-Gyorgyi, A. (1939). Quoted by Theorell, H. & Swedin, B. (1939). *Naturwissenschaften*, **27**, 95.
- Theorell, H. & Swedin, B. (1939). *Naturwissenschaften*, **27**, 95.
- Thurlow, S. (1925). *Biochem. J.* **19**, 175.
- Vennesland, B., Ceithaml, J. & Gollub, M. C. (1947). *J. biol. Chem.* **171**, 445.
- Vennesland, B. & Evans, E. A. (1944). *J. biol. Chem.* **156**, 783.
- Vennesland, B., Evans, E. A. & Francis, A. M. (1946). *J. biol. Chem.* **163**, 573.
- Vennesland, B. & Felsher, R. Z. (1946). *Arch. Biochem.* **11**, 279.
- Willstätter, R. & Stoll, A. (1918). *Liebigs Ann.* **416**, 21.